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FULL PAPER

MICA and recovery from hepatitis C virus and hepatitis B virus infections

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The polymorphic MHC class I chain-related A (MICA) gene encodes a ligand that has different binding affinities for the NKG2D activating receptor of CD8+ T cells and natural killer (NK) cells. We hypothesized that MICA heterogeneity would affect recovery from hepatitis C virus (HCV) and hepatitis B virus (HBV) infections. To test the hypothesis, we initially typed known MICA polymorphisms for 228 persons who cleared HCV infection and 442 persons with persistent hepatitis C matched on other factors affecting viral persistence. Although MICA*015 was detected more than two-fold more often in persons with viral clearance (odds ratio 0.36, 95% confidence interval = 0.19, 0.80), it occurred in fewer than 5% of the study population. In a similar analysis of 442 persons with chronic hepatitis B and 768 matched controls who recovered, MICA*015 was detected in 2.0% of persons with chronic hepatitis B and only 0.9% of controls. No significant associations were detected with other MICA polymorphisms. While further investigation may reveal a structural basis of the MICA*015 associations, these data provide little support for the hypothesis that differential distribution of MICA alleles substantially affects recovery from HCV and HBV infections.
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Introduction

Worldwide, an estimated 170 million persons are infected with hepatitis C virus (HCV), which in persons with persistent infection can cause cirrhosis and hepatocellular carcinoma.[1–4] There is a substantial amount of evidence suggesting that host genetic differences influence recovery from HCV infection. Even when a large number of persons have been accidentally infected by the same viral inoculum, some recover while others develop viral persistence.[5,6] In addition, recovery from HCV infection occurs more often in Caucasians than African Americans, even after adjusting for environmental and viral factors.[7] Some host genetic polymorphisms in the human leukocyte antigen complex have been associated with HCV recovery.[8,9] However, much of the genetic basis for viral recovery remains unknown.

Although the mechanisms of recovery from HCV infection remain poorly understood, early broad and vigorous cellular anti-HCV immune responses are associated with HCV clearance.[10] There is evidence that natural killer (NK) cells contribute to HCV recovery,[11] and conversely that, at least in vitro, HCV may persist by interfering with NK activation.[12,13] How HCV infection promotes or inhibits activation of NK cells, and why there are person-to-person differences in the immune response are poorly understood.

Worldwide, there are more than 300 million persons infected with hepatitis B virus (HBV), which can cause chronic liver disease and hepatocellular cancer.[14,15] As with HCV infection, the host immune response to HBV infection determines viral persistence,[16,17] which has been associated with polymorphisms in the human leukocyte antigen complex and other immune response genes.[18–24] Although there are likely important differences in the mechanisms of HBV persistence, there is also evidence that NK T (NKT) cells play an important role.[25,26]

The nonclassical MHC class I chain-related A (MICA) gene encodes a protein that functions as a ligand for NKG2D, an activating receptor on macrophages, CD8+ T cells, γδ T cells, NKT cells, and natural killer NK cells.[27,28] MICA is highly polymorphic, and there is evidence that the various allotypes bind to NKG2D with different affinities.[29] Thus, it is tenable that affinity differences might result in different degrees of activation.

MICA is expressed on gastroepithelial and vascular endothelial cells in response to various factors such as heat, UV light, or microbial infection.[28,30–33] MICA expression also occurs in certain carcinomas, and has been found in hepatocellular cancer tissue and hepatoma cell lines.[34] In addition, in one study, MICA induction on dendritic cells was strongly impaired in persons with persistent HCV infection, compared with healthy controls.[35] However, whether genetic differences in MICA contribute to HCV and HBV recovery is unknown.

To investigate whether MICA polymorphisms are associated with HCV recovery, we compared the frequency of all known MICA polymorphisms in two large, well-characterized panels of persons who recovered from either HCV or HBV infection and controls matched on nongenetic factors.

Results

Study cohorts
The HCV study group comprised 228 clearance subjects and 442 persistently infected subjects (for 14 subjects only one control was available) carrying 456 and 884 alleles, respectively. No significant differences were detected between cases and controls with respect to matching criteria (HIV status, gender, and race, data not shown). However, a greater proportion of persons had chronic HBV infection in the HCV clearance group (15.0%) compared to those with HCV persistence (5.1%) ($P < 0.001$). The HBV study group comprised 221 persons with chronic hepatitis B and 384 persons who had

recovered from HBV infection (58 persons had only one control), for a total of 442 and 768 alleles, respectively. No significant differences were detected between the two HBV study groups with respect to matching criteria (human immunodeficiency virus (HIV) status, age, and gender).

MICA*015 and HCV clearance
Of the 27 unique MICA polymorphisms examined, only one was detected more often in persons who had cleared HCV infection, compared with controls (Table 1). MICA*015 was detected more than twice as often in persons with viral clearance as in those with persistent infection ($P = 0.01$). However, only 3.9% of all those examined with viral clearance were MICA*015 positive. In addition, the allele was too rare in Whites (0.3%) to be examined in this subset (Table 2). Consequently, the association detected in the overall group was entirely due to a strong association of MICA*015 with HCV clearance in a small number of Blacks.

The MICA gene is 46.5 kb from the MHC class I HLA-B gene so we used HLA results from one of our previous studies[8] to see if MICA*015 is in linkage disequilibrium with any class I alleles that may also be associated with HCV clearance. In Blacks, MICA*015 was in linkage disequilibrium with B*4501 ($P = 0.03$). However, it did not appear that the linkage with B*4501 was the explanation for the association with MICA*015, since removal of all B*4501 positive persons from analysis did not significantly alter the MICA*015 association (odds ratio (OR) = 0.36 with B*4501 removed and 0.39 with all included).

Table 1 MICA polymorphisms and recovery from hepatitis C infection

MICA allele	HCV status (% with allele)		OR	95% CI	P-value
	Recovered (n = 456)	Persistent (n = 884)			
High affinity[a]					
*001	0.9	1.9	2.45	0.81, 7.43	0.11
*00201	15.6	18.1	1.19	0.87, 1.63	>0.20
*00701	3.9	2.5	0.61	0.31, 1.19	0.15
*011	2.2	2.9	1.35	0.64, 12.87	>0.20
*01201	1.5	1.8	1.22	0.50, 3.01	>0.20
*015	3.9	1.7	0.39	0.19, 0.80	0.01
*017	3.3	2.0	0.58	0.28, 1.18	0.13
*018	3.7	2.8	0.74	0.40, 1.39	>0.20
*041	0.2	0.9	4.00	0.50, 31.93	0.19
Low affinity[a]					
*004	11.8	14.3	1.22	0.87, 1.72	>0.20
*00801	34.2	35.0	1.05	0.83, 1.35	>0.20
*00802	2.2	1.9	0.86	0.38, 1.95	>0.20
*00901	4.8	4.4	0.91	0.53, 1.57	>0.20
*00902	2.4	1.8	0.76	0.35, 1.66	>0.20
*010	4.6	4.3	0.93	0.54, 1.62	>0.20
*016	1.3	1.1	0.87	0.30, 2.46	>0.20
*019	1.1	0.9	0.79	0.25, 2.47	>0.20

[a]Binding affinities to NKG2D were determined by Steinle et al[29] for MICA*001, *00701, *00702, *004, *00801, *00802, and *016 and, attributed to the amino-acid position at 129 in the MICA gene. Based on amino-acid composition (methionine, high; valine, low), affinities were projected for the other alleles.

Note. n is the number of alleles examined in persons who either had cleared infection or had persistent infection. OR, 95% CI and P-values were calculated using conditional logistic regression. OR represents the likelihood of being persistently HCV infected if carrying a specific allele. Also tested but not shown were the following alleles that were detected in a total fewer than five cases and controls: MICA*00202, *006, *00702, *021, *030, *044, *045, *046, *N1 (a novel allele detected by an unusual SSO hybridization pattern).

Table 2 MICA*015 and recovery from hepatitis C by race

Study subjects	Frequency of clearance (n), %	Frequency of persistence (n), %	OR (95% CI)	P-value
All subjects	884	456		
% positive	3.9	1.7	0.39 (0.19, 0.80)	0.01
White	430	230		
% positive	0	0.15	NA	NA
Black	360	182		
% positive	8.8	3.3	0.35 (0.16, 0.76)	0.008

Note. n is the no. of alleles examined in persons who either had cleared infection or had persistent infection. OR, odds ratio; CI, confidence interval.

MICA binding to NKG2D

Steinle et al[29] demonstrated that MICA*001 and *007 have high binding affinity to NKG2D, while MICA*004, *008, and *016 have low-binding affinity. Using a recessive model, we compared individuals who had two copies of any of the three low-affinity alleles MICA*004, *008, and *016 to individuals with at least one copy of the high-affinity alleles MICA*001 and *007. We did not detect any difference in the odds of HCV persistence in persons with two low binding alleles compared to the others (P < 0.2). Since binding affinity was attributed to the amino acid residue at position 129 (see Methods), we also classified the binding of all MICA allotypes according to amino-acid residue that repeated the same analysis. Although MICA*015 is assigned to the high-affinity

group, no association of this grouping was detected with HCV persistence (P > 0.20).

MICA binding and hepatitis B

We also examined the frequency of all MICA polymorphisms in a previously described panel of 442 subjects with chronic hepatitis B, and 768 matched controls.[36] MICA*015 was detected 2.6 fold more often in persons with chronic hepatitis B, but the allele was uncommon (2.0%) even in these individuals, and rare in those who had recovered from chronic hepatitis B (0.9%) (P = 0.072) (Table 3). No other associations between MICA polymorphisms and clearance of HBV infection approached statistical significance. No association was detected between the binding affinity of MICA and HBV persistence in analyses that were analogous to those performed for HCV (data not shown).

Discussion

Despite the recently described importance of innate immunity in recovery from viral infections and the unfolding role of MICA in NK cell activation, this is the first study we are aware of that investigated whether MICA genetic polymorphisms explain the highly variable infection outcomes. Using very large panels of well characterized patient cohorts, we detected associations of MICA*015 with recovery from HCV infection and, conversely, with chronic hepatitis B. While these findings need to be confirmed and explained, MICA*015 only was detected in a small fraction of persons with these two outcomes, and no other significant associations were

Table 3 MICA polymorphisms and recovery from hepatitis B infection

MICA allele	HBV status (% with allele)		OR	95% CI	P-value
	Recovered (n = 768)	Chronic HBV (n = 442)			
High affinity[a]					
*001	1.3	1.6	1.14	0.42, 3.11	> 0.20
*00201	14.8	13.3	0.92	0.65, 1.29	> 0.20
*00701	3.4	2.7	0.76	0.37, 1.55	> 0.20
*011	3.8	3.2	0.85	0.44, 1.64	> 0.20
*01201	1.2	0.90	0.63	0.19, 2.08	> 0.20
*015	0.9	2.04	2.66	0.92, 7.71	0.07
*017	2.6	3.4	1.23	0.63, 2.49	> 0.20
*018	3.0	4.8	1.61	0.87, 2.97	0.13
Low affinity[a]					
*004	9.9	12.0	1.29	0.89, 1.87	0.18
*00801	38.0	39.6	1.06	0.83, 1.35	> 0.20
*00802	1.0	1.4	1.22	0.42, 3.58	> 0.20
*00901	6.6	5.0	0.76	0.45, 1.27	> 0.20
*00902	1.9	0.7	0.35	0.10, 1.24	> 0.10
*010	4.4	5.0	1.09	0.62, 1.91	> 0.20
*016	3.0	2.0	0.66	0.29, 1.47	> 0.20
*019	1.2	1.6	1.25	0.45, 3.46	> 0.20

[a]Binding affinities to NKG2D were determined by Steinle et al[29] for MICA*001, *00701, *00702, *004, *00801, *00802, and *016 and attributed to the amino acid position at 129 in the MICA gene. Based on amino-acid composition, affinities were projected for the other alleles.
Note. n is the number of alleles examined in persons who either had cleared infection or had persistent infection. OR, 95% confidence intervals (CI) and P-values were calculated using conditional logistic regression. OR represents the likelihood of being persistently HCV infected if carrying a specific allele. Also tested but not shown were the following alleles that were detected in a total fewer than five cases and controls: MICA*00202, *006, *00702, *021, *030, *041, *044, *045, *046, *N1.

detected. Collectively, these data suggest that MICA heterogeneity does not substantially contribute to recovery from hepatitis virus infections.

Lymphocytes that express NKG2D (CD8 + T cells, γδ T cells, NKT cells, and NK cells) are present in high concentrations in the liver, and there is evidence that both active CD8^{+} T-cell and NK cell immune responses are important for HCV clearance.[10,11] However, the nature of MICA expression in response to HCV infection in the liver is not clear. Hepatoma cells express MICA,[34] and MICA-expressing hepatocytes may augment the immune response to HCV infection. Interferon-α may also induce in vitro NK activation of MICA on dendritic cells, a process that appears to be impaired in dendritic cells taken from persons with chronic hepatitis C.[35] If HCV has developed mechanisms of persistence that over-ride MICA-mediated immune activation, these generally negative findings would not be surprising. Moreover, it would be interesting to see if persons with chronic hepatitis C who respond to interferon-α-based treatment are more likely to be MICA*015 positive than those who do not.

Another important possibility is that MICA polymorphism does not affect function of the allotypes. NKG2D binding affinity is the only phenotypic distinction that has been made for MICA allotypes and it is possible that binding affinities of both the low- and high-affinity groups are well within the range of promoting an NKG2D-mediated response. This conjecture is compatible with the lack of overall associations between group assignments based on binding affinity and HCV/HBV outcomes in this study.

Only MICA*015 appeared to have any association with recovery from HBV infection as well. Although this association could have occurred by chance, notably, the direction of the association was opposite to that for HCV infection. In particular, MICA*015 was associated with HCV clearance and HBV persistence. Since persons with chronic hepatitis B are over-represented in the HCV clearance group, another important consideration is that MICA*015 is primarily associated with HBV persistence (or with some other linked HBV persistence allele), and only indirectly with HCV clearance. However, this seems unlikely because removal of persons with chronic hepatitis B from the analysis did not alter the strength of the MICA*015 association with HCV clearance.

There are a few limitations to our data. As mentioned above, there were too few MICA*015 positive persons to allow us to assess whether the association with HCV clearance and HBV persistence were independent. Also, we cannot exclude the possibility that MICA*015 is tightly linked to another allele that is responsible for the association. We did not detect linkage between MICA*015 and either HLA-B or TNF, the most plausible candidates based on existing studies. In addition, we cannot exclude chance as an explanation for the associations since the levels of statistical significance would be diminished by correction for multiple comparisons. Conversely, valid associations of some very low frequency alleles with HCV and HBV clearance may have been missed. However, we can be confident that the described polymorphisms in MICA contributed little to persistence of HBV and HCV infections.

Methods

Study participants

Subjects in this study were participants in one of the four studies: (i) AIDS Link to Intravenous Experience (ALIVE) Study, which is an ongoing study of 2921 injection drug users enrolled in Baltimore, MD, from February 1988 to March 1989, as previously described;[37] (ii) Multicenter Hemophilia Cohort Study (MHCS), which is a prospectively followed cohort of patients with hemophilia, von Willebrand's disease, or a related coagulation disorder from 16 comprehensive hemophilia treatment centers enrolled between 1982 and 1996, as previously described;[38] (iii) Hemophilia Growth and Development Study (HGDS), which is a continuing study of 333 children and adolescents with hemophilia enrolled between March 1989 and May 1990, as previously described;[39] or (iv) Multicenter AIDS Cohort Study (MACS), which is an ongoing study of 5622 gay men enrolled in one of the four United States cities between 1984–1985 and between 1987–1991, as previously described.[40,41]

To investigate the hypothesis that MICA may be associated with HCV and HBV clearance, a nested case–control design was used with cases defined as the least common event, viral clearance in the case of HCV infection and viral persistence in the case of HBV infection. All available persons who met the definition of a case were selected, and, to increase the power of the study, matched to two controls, which were available in excess. For HCV, cases had cleared prior infection as demonstrated by an inability to detect HCV RNA in at least two serum samples that were drawn a minimum of 6 months apart. Prior infection was substantiated by detection of HCV antibody (anti-HCV). Persistently infected control individuals were selected from the same cohort and had anti-HCV and HCV RNA in serum for a minimum of 6 months. Controls were matched 2 : 1 to individuals in the same cohort by HIV status, gender, geographic location (if applicable), and race. These factors were chosen because HIV status and race are determinants of viral clearance.[7]

For HBV, cases were persistently infected with HBV and matched to two controls from the same cohort based on factors that have been associated with HBV outcomes, including age within 10 years, gender, and HIV type-1 (HIV-1) status. Subjects were considered persistently infected with HBV if they tested positive for hepatitis B surface antigen (HBsAg) at two visits separated by a minimum of 6 months. Testing for antibodies against hepatitis B core antigen (anti-HBc) and HBsAg (anti-HBs) was performed as needed to exclude primary HBV infection. Individuals with viral clearance (controls) were positive for anti-HBc and anti-HBs without the presence of HBsAg at two visits separated by a minimum of 6 months. HBV status of the HIV-positive subjects was defined before highly active antiretroviral therapy was available. Informed consent was obtained from all participants and the study was approved by the institutional review boards at all participating institutions.

Serologic HCV testing

Subjects who tested positive for anti-HCV by second-generation Ortho HCV 2.0 enzyme immunoassay (EIA)

(Ortho Diagnostic Systems, Raritan, NJ, USA) had two samples, taken at least 6 months apart, assessed for HCV RNA by a branched DNA (bDNA) assay (Quantiplex HCV RNA 2.0 assay; Chiron Corporation, Emeryville, CA, USA). Subjects with two samples below the limit of detection by the bDNA assay had at least one of the two samples retested with the HCV COBAS AMPLICOR system (COBAS AMPLICOR HCV; Roche Diagnostics, Branchburg, NJ, USA), and their antibody status was confirmed by a recombinant immunoblot assay (RIBA 3.0) (Chiron Corporation). Only subjects with a negative HCV RNA result confirmed by COBAS testing were considered to have cleared infection. Subjects with positive bDNA assay results were eligible to be matched to the HCV clearance subjects as controls. All assays were performed according to the manufacturer's specifications except for COBAS testing of samples that contained heparin. These samples were treated with heparinase prior to COBAS testing using a protocol developed by the manufacturer. All samples used for testing had been stored at -70°C after processing, and had not been previously used for other assays.

HIV and HBV serologic testing

HIV-1 testing was done by EIA, and positive results for specimens were confirmed by Western blotting as previously reported.[36–38] Testing for HBV including hepatitis B surface antigen (HBsAg), antibody to hepatitis B core (anti-HBc), and antibody to HBsAg (anti-HBs) was performed using commercially available enzyme immune assays according to specifications (Abbott Laboratories, Abbott Park, IL, USA).

MICA typing

An Epstein–Barr virus transformed cell line was established for each subject, and DNA was extracted from these cell lines by phenol–chloroform extraction. High-resolution (allele level) MICA genotyping was performed using a sequence specific oligonucleotide (SSO) protocol locally developed on the basis of DNA sequences of known MICA alleles (http://www.anthonynolan.com/HIG/seq/nuc/text/mica_nt.txt) and a previous publication on MICA typing.[42] MICA was amplified using locus-specific PCR primers flanking exons 2 and 4 to characterize the polymorphisms present in exons 2, 3, and 4. Each individual exon is then separately amplified from the first PCR product using nested primers that flank each exon. The primers used for exon 2 were forward 5′-TCTTGTCCCTTTGCC CGTGTGCAT-3′, reverse 5′-CCCCCATTCCTCACCCC CAGCCTG-3′; exon 3 were forward 5′-TGGGGGA GGGCCAGGGAGGCGTAC-3′, reverse 5′-CGATGTGC CAACAGGAAATGCCTT-3′; and exon 4 were forward 5′-CAGACTTGCAGGTCAGGGGTCCCG-3′, and reverse 5′-CAATGACTCTGAAGCACCAGCACT-3′. The PCR products from each exon were then blotted on N$^+$ nylon membranes and hybridized with a panel of SSO probes. MICA alleles were assigned to the individuals by the reaction patterns of the SSO probes based on the known MICA sequences.

Statistical analysis

All analyses were performed using SAS version 6.12 (SAS Institute, Cary, NC, USA). The frequencies of MICA alleles were compared between those who cleared HCV infection and those who had a persistent infection. OR were determined by univariate and multivariate conditional logistic regression, and reflect the likelihood of persons carrying a particular allele being persistently infected with either HCV or HBV. Linkage disequilibrium values between individual MICA alleles and HLA-B alleles (previously typed)[8] were generated using the standard relative disequilibrium (rD) calculations. To analyze the effect of strong *vs* weak MICA binding affinity for NKG2D, we grouped together the allele frequencies of MICA*001 and *007 as strong binders and MICA*004, *008, and *016 as weak binders according the MICA/NKG2D binding affinity results described by Steinle *et al*.[29] In this analysis, a recessive model for the low-affinity ligands was used in which individuals carrying two copies of any combination of MICA*004, *008, and *016 were compared with individuals carrying at least one copy of either MICA*001 and/or *007, based on the hypothesis that a phenotypic (functional) difference would most likely be expected for those with no high affinity binding alleles. In a second analysis, all alleles with methionine at position 129 of the MICA allele were considered 'high binders' and those with valine, 'low binders'. Individuals with two 'low' affinity residues were compared to all others (ie those with at least one high-affinity allele).

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